Effect of heat shock protein 70 modulators on the development of morphine analgesic tolerance in rats

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The clinical use of opioid analgesics, such as morphine, is limited by analgesic tolerance, molecular mechanism of which is not well understood. Recently, molecular chaperone heat shock protein 70 (Hsp70) has been demonstrated to play important roles in morphine-induced neuroadaptation. Here, we focused on the involvement of Hsp70 in the development of analgesic tolerance to morphine. Rats were treated with morphine (5, 10, 20 mg/kg, subcutaneously) or saline once daily for 10 consecutive days. Hsp70 modulator *N*-formyl-3, 4-methylenedioxybenzylidine-γ-butyrolactam [KNK437, 100 mg/kg, intraperitoneally (i.p.)], geranylgeranylacetone (500 mg/kg, i.p.) or pifithrin-μ (20 mg/kg, i.p.) was administered before morphine (10 mg/kg, subcutaneously)/saline treatment. Analgesic effect of morphine was measured using the tail-flick latency test, and Hsp70 protein expression was examined by western blot. Analgesic effect of morphine decreased gradually with the increase in the number of days of morphine injection, indicating development of analgesic tolerance. A significant increase of Hsp70 expression in the periaqueductal gray was observed during the development of analgesic tolerance after repeated morphine injections. The development of morphine analgesic tolerance was suppressed by pre-treatment with Hsp70 transcriptional inhibitor KNK437 or functional antagonist pifithrin-μ, while promoted by pre-treatment with Hsp70 transcriptional inducer geranylgeranylacetone. Our results demonstrated that the development of morphine analgesic tolerance was dual regulated by Hsp70 modulators, suggesting Hsp70 as an interesting and new target for preventing the development of opioid analgesic tolerance. ***Behavioural Pharmacology*** **31: 179–185 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Analgesic tolerance refers to the decrease of analgesic effect after repeated exposure to opioids and is a major barrier to clinical application of morphine and related drugs (Jamison and Mao, 2015). Escalation of opioid doses is always required to obtain the equivalent effect after tolerance, which may cause a lot of adverse effects and social problems (Mao, 2006; Khademi *et al*., 2016). Numerous evidence has demonstrated adaptive changes in the periaqueductal gray (PAG) to be essential for the development of opioid analgesic tolerance, such as activation of astrocytes, increase of phosphatase activity and reduction in mu-opioid receptor signaling efficacy (Bagley *et al*., 2005; Gabra *et al*., 2007; Harada *et al*., 2013; Wang *et al*., 2017). However, the underlying mechanism is still not fully understood, and so far, there is no effective way to manage opioid analgesic tolerance (Xu *et al*., 2014).

Heat shock protein 70 (Hsp70) is a major member of molecular chaperones, a class of proteins helping other proteins to acquire their functionally mature constructions (Hartl *et al*., 2011). Recently, Hsp70 has been the focus of attention for its neuroprotective effect in some neurodegenerative diseases, such as Alzheimer's disease (Evans *et al*., 2010; Boiocchi *et al*., 2015; Marino Gammazza *et al*., 2016). The possible mechanism of Hsp70 in the pharmacological action of opioids has been widely investigated for decades (Oliveira *et al*., 2002; Ammon-Treiber and Hollt, 2005; Yang *et al*., 2014). Our previous studies demonstrated that Hsp70 in the nucleus accumbens plays an important role in neuroadaptation associated with opioid addiction (Luo *et al*., 2011; Qin *et al*., 2013; Wang *et al*., 2014; Qin *et al*., 2016). However, the involvement of Hsp70 in the development of opioid analgesic tolerance has not been fully characterized.

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The present study investigated the effect of Hsp70 modulators on the development of morphine analgesic tolerance using pharmacological approaches. Research results will further expand our knowledge in the role of Hsp70 in pharmacological function of opioids and provide a potential treatment target for opioid analgesic tolerance.

Methods

Subjects

A total of 115 Sprague–Dawley rats (Vital River Laboratory Animal Technology Co., Ltd., China), initially weighing 200 to 220 g, were used in this study. The rats were housed in transparent plastic cages (4–6 rats per cage) and kept in a light (12 h light/12 h dark, 08:00 hours light on), temperature (22 ± 1°C) and relative air humidity (50 ± 10%) controlled environment with freely access to food and water. Experiments were carried out during the light cycle after rats were habituated to the housing conditions for at least 5 days, habituated to the tail-flick assay and handled for at least 3 days. All experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The animal care and experimental procedures were approved by the local Committee of Animal Care and Use.

Drugs

Morphine hydrochloride (Morphine) was obtained from Qinghai Pharmaceutical Manufactory (Xining, China). *N*-formyl-3, 4-methylenedioxybenzylidine-γ-butyrolactam (KNK437), geranylgeranylacetone and pifithrin-μ were purchased from Calbiochem KGaA (Darmstadt, Germany), Sigma Chemical Co. (St.Louis, Missouri, USA) and Tocris Bioscience (Bristol, UK), respectively. Morphine was dissolved in 0.9% saline, and geranylgeranylacetone, pifithrin-μ and KNK437 were dissolved in olive oil (olive). All drugs were freshly prepared immediately before experiments and administered with the volumes of 0.1 ml/100 g. Drugs were administered intraperitoneally (i.p.) except morphine, which was administered subcutaneously.

Tail-flick latency test

A tail-flick assay (Analgesia Meter: Ugo Basile, Italy) was used to measure the tail-flick latency. Briefly, a beam of light was focused on the dorsal tail surface approximately 2 cm from the tip of the tail, and the tail-flick latency was defined by a rapid tail-flick response. The intensity of the light was adjusted to ensure that baseline flick latency of rats was 2 to 3 s. A cutoff time of 12 s was used to avoid tissue damage. Tail-flick latency was measured for 3 times with an intertrial interval of 1 min, and the mean of three results was taken as the final latency. Baseline latency and postdrug tail-flick latency were determined for each rat 30 min before and after morphine/saline injection, respectively. Tail-flick response latency (s) was expressed as the percentage of maximal possible effect (%MPE) using the equation below: %MPE = [Postdrug latency (s) – Baseline latency (s)]/[Cutoff value (s) – Baseline latency (s)] × 100%. Morphine analgesic tolerance was evaluated by disappearance of differences in %MPE between control group (saline treatment) and experimental group (morphine treatment). Baseline latencies among different groups of each experiment or different test days of each group did not vary significantly when analyzed by one-way analysis of variance (ANOVA), indicating no tissue damage or difference in pain threshold which could influence the results.

Western blot

The PAG was quickly removed from coronal slices containing the PAG, which were prepared according to The Rat Brain in Stereotaxic Coordinates (sixth edition) (Paxinos and Watson, 2007). Total protein was extracted by homogenizing the PAG tissue in ice-cold radio-immunoprecipitation assay lysis buffer, and protein content was measured using bicinchoninic acid protein assay kit (Applygen Technologies Inc., Beijing, China). Protein samples were separated by electrophoresis on SDS polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were washed with TBS/T (Tris-buffered saline mixed with 0.05% Tween-20) containing 5% nonfat dry milk for 1 h to block nonspecific antibody binding sites, incubated with antibodies against Hsp70 protein (R&D, Minneapolis, Minnesota, USA) or β-actin (Santa Cruz, California, USA) at 4°C overnight and then treated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, California, USA) for 1 h at room temperature. The blots were probed by chemiluminescent detection method (Applygen Technologies Inc. Beijing, China) and then exposed to X-ray films. The content of the Hsp70 protein in blots was quantified by a Gel Doc 2000 densitometer (Bio-Rad, Hercules, California, USA) and normalized to signals of β-actin protein.

Statistical analysis

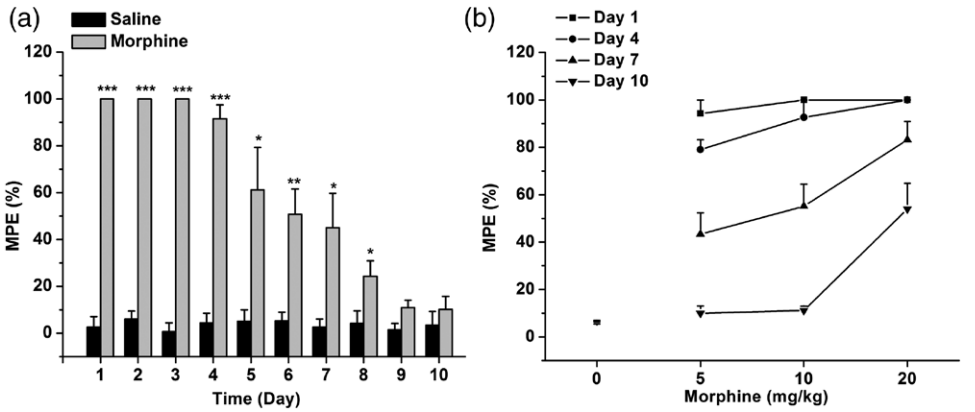
The data in Figs 1b, 3a, 4a and 5a were analyzed by two-factor repeated-measures ANOVA followed by *post hoc* Bonferroni test. An independent-samples *t* test was applied to determine differences between the two groups on each day in Fig. 1a. The area under the curve (AUC) was calculated by the trapezoid area formula for each rat and then analyzed by two-way ANOVA followed by an independent-samples *t* test. The data in Fig. 2 were analyzed by one-way ANOVA and *post hoc* Bonferroni test. The significant level was taken as $P < 0.05$.

Results

Tolerance to the analgesic effect induced by morphine injection once daily

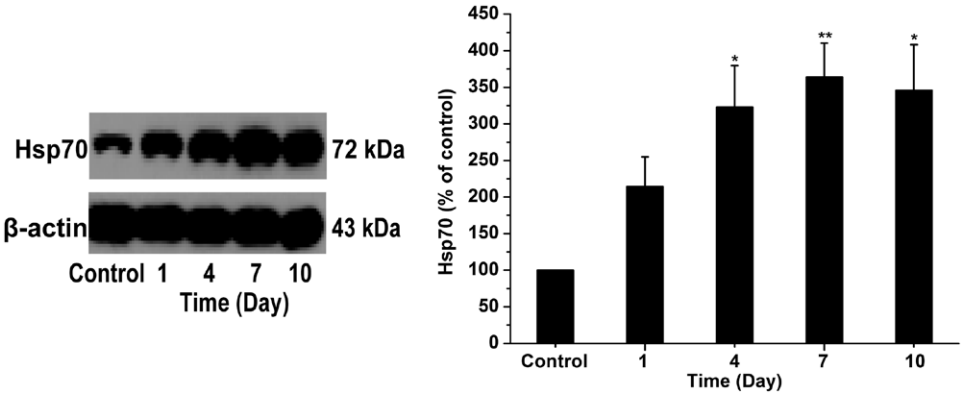
In order to evaluate morphine analgesic tolerance, rats were injected with saline or morphine (10 mg/kg,

Fig. 1



Analgesic effect of morphine (10 mg/kg, subcutaneously) injected once daily and its dose–effect curve. (a) Analgesic tolerance developed on the 9th and 10th days as there was no significant difference in percentage of maximal possible effect (%MPE) between the Morphine group and the Saline group. (b) A significant decrease of morphine analgesic effect after repeated exposures. Data are expressed as means ± standard error of the mean (SEM). n=5. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ vs. corresponding Saline group.

Fig. 2



Heat shock protein 70 (Hsp70) protein expression in the periaqueductal gray (PAG) on different days after repeated morphine treatments once daily. Hsp70 level rose after morphine exposure, and significant increase was observed on days 4, 7 and 10 (n=5). Data normalized to the control group (taken as 100%) are expressed as means ± standard error of the mean (SEM). $^{*}P<0.05$, $^{**}P<0.01$ vs. Control group.

subcutaneously) once daily for 10 consecutive days. Baseline latency and postdrug latency were measured before and after each injection, respectively. As shown in Fig. 1a, the analgesia effect of morphine was reduced within 4 days and had no significant difference when compared with that of saline on days 9 and 10, which demonstrated the development of analgesic tolerance.

To demonstrate analgesic tolerance from another aspect, rats were treated with saline or morphine (5, 10, 20 mg/kg, subcutaneously) once daily for 10 consecutive days and tail-flick latency was measured on days 1, 4, 7 and 10. Fig. 1b shows the dose–response curves on different days after morphine injection once daily. A two-factor repeated-measures ANOVA demonstrated a significant effect for treatment days (days: $F_{3,16}=120.322$, $P<0.001$), indicating a significant decrease of analgesic effect following repeated morphine injections.

Time-dependent Hsp70 expression in the PAG during the development of morphine analgesic tolerance

To assess whether Hsp70 protein is involved in the development of analgesic tolerance, we firstly examined the expression level of Hsp70 in the PAG on different days after morphine injection (10 mg/kg, subcutaneously) once daily. As shown in Fig. 2, Hsp70 protein expression significantly increased after morphine injection once daily for no less than 4 days (one-way ANOVA: $F_{4,24}=5.678$, $P<0.01$; Bonferroni's test: $P<0.05$, 0.01 and 0.05 vs. Control group for days 4, 7 and 10, respectively). However, there was no significant difference in Hsp70 expression among days 4, 7 and 10. These results indicate that Hsp70 expression in

PAG increases rapidly within the first 4 days and then maintains at a high level during the development of analgesic tolerance induced by morphine injection once daily.

Effect of Hsp70 modulators on the development of morphine analgesic tolerance

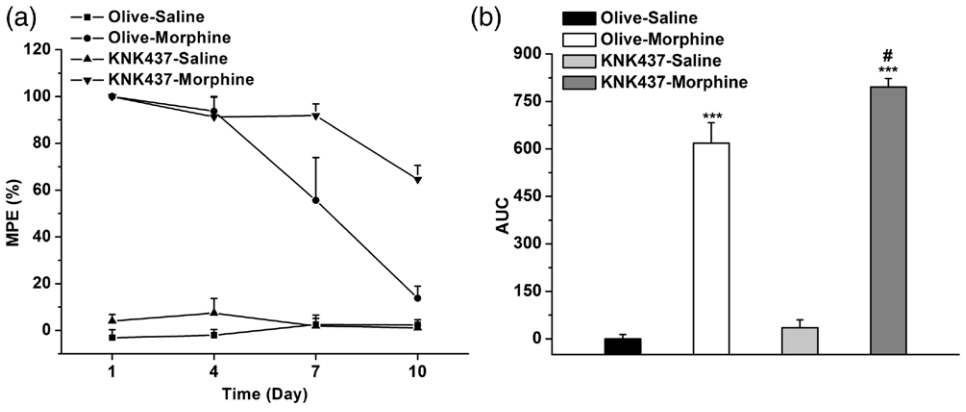
To further evaluate the possible role of Hsp70 in morphine analgesic tolerance, we examined the effect of different Hsp70 modulators on the development of morphine analgesic tolerance.

The first Hsp70 modulator we employed is KNK437, an Hsp70 transcriptional inhibitor. KNK437 (100 mg/kg, i.p.) or vehicle was administered 6 h before each morphine (10 mg/kg, subcutaneously) injection for 10 consecutive days, and tail-flick latency was measured on days 1, 4, 7 and 10. As shown in Fig. 3a, repeated administration of KNK437 did not significantly alter pain threshold of rats, as there was no significant difference between Olive–Saline group and KNK437–Saline group. Also, no significant difference was observed between Olive–Morphine group and KNK437–Morphine group on day 1. However, after repeated administration before morphine for 10 consecutive days, KNK437 significantly delayed the decrease of analgesic effect of morphine (groups: $F_{3,16} = 177.524$, $P < 0.001$; Bonferroni test: $P < 0.01$ vs. Olive–Morphine group). The above data were further analyzed in form of area under the curve (AUC) in Fig. 3b (KNK437: $F_{1,16} = 8.032$, $P < 0.05$; Morphine: $F_{1,16} = 337.117$, $P < 0.001$; KNK437 × Morphine: $F_{1,16} = 3.583$, $P > 0.05$). There was no significant difference between KNK437–Saline and Olive–Saline group in AUC (1–10 days) values, whereas pretreatment with KNK437 prior to morphine significantly increased the AUC (1–10 days) values when compared with Olive–Morphine group ($P < 0.05$).

Geranylgeranylacetone, another Hsp70 modulator employed, is an Hsp70 transcriptional inducer. Geranylgeranylacetone (500 mg/kg, i.p.) or vehicle was administered 24 h before each morphine (10 mg/kg, subcutaneously) injection for 10 consecutive days, and tail-flick latency was measured on days 1, 4, 7 and 10. On day 1, no significant difference was observed between Olive–Saline group and geranylgeranylacetone–Saline group nor between Olive–Morphine group and geranylgeranylacetone–Morphine group. After repeated pretreatments for 10 consecutive days, geranylgeranylacetone made no significant difference on pain threshold between Olive–Saline group and geranylgeranylacetone–Saline group but significantly quicken the decrease of morphine analgesic effect in rats of geranylgeranylacetone–Morphine group (groups: $F_{3,16} = 118.877$, $P < 0.001$; Bonferroni test: $P < 0.05$ vs. Olive–Morphine group) (Fig. 4a). The effect of geranylgeranylacetone was also analyzed in the form of AUC (geranylgeranylacetone: $F_{1,16} = 5.325$, $P < 0.05$; morphine: $F_{1,16} = 220.369$, $P < 0.001$; geranylgeranylacetone × morphine: $F_{1,16} = 5.966$, $P < 0.05$) (Fig. 4b). A significant difference was found between Olive–Morphine group and geranylgeranylacetone–Morphine group in AUC (1–10 days) values ($P < 0.05$).

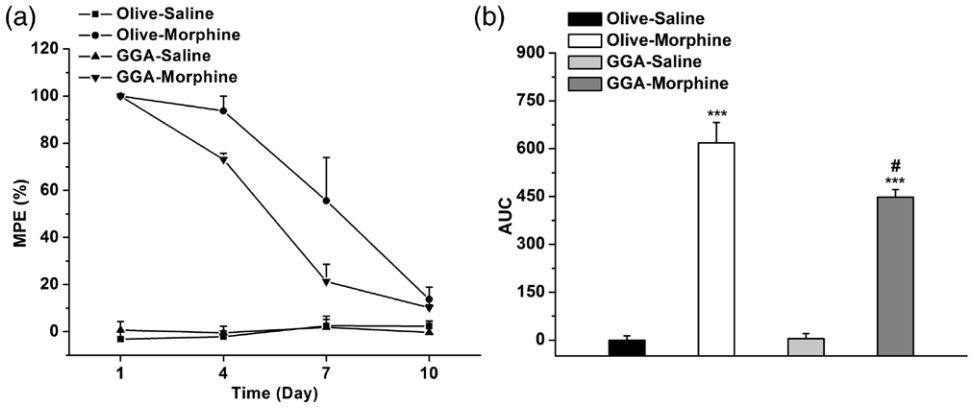
We also examined the effect of functional antagonism of Hsp70 by pifithrin-μ (20 mg/kg, i.p.) on the development of morphine analgesic tolerance. As shown in Fig. 5a, the MPE of rats treated with pifithrin-μ and morphine was significantly greater than that of rats treated with olive oil and morphine (groups: $F_{3,16} = 180.432$, $P < 0.001$; Bonferroni test: $P < 0.01$ vs. Olive–Morphine group). The AUC (1–10 days) value of pifithrin-μ–Morphine group was significantly higher than that of Olive–Morphine ($P < 0.05$), while there was no significant difference between Olive–Saline group and pifithrin-μ–Saline group

Fig. 3



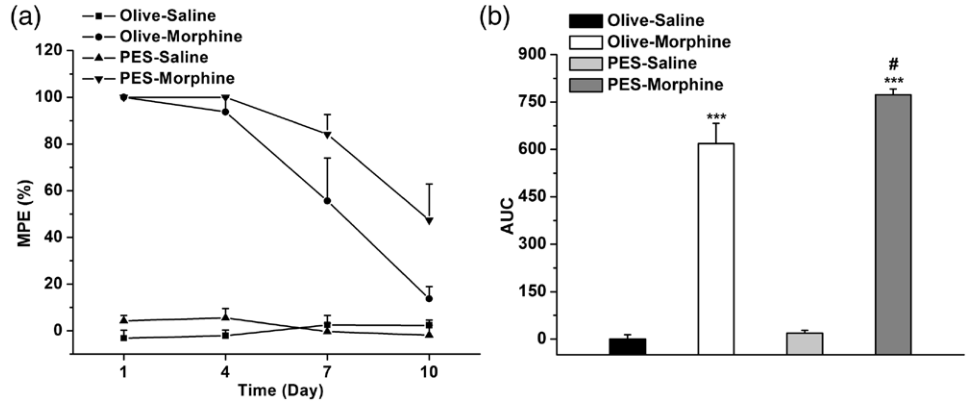
Effect of *N*-formyl-3, 4-methylenedioxybenzylidine-γ-butyrolactam (KNK437) [100 mg/kg, intraperitoneally (i.p.)] on the development of morphine analgesic tolerance represented by maximal possible effect (MPE) (a) and area under the curve (AUC) (b). KNK437 given 6 h before each morphine injection delayed the development of analgesic tolerance and improved the analgesic efficacy of morphine (n=5). Data are expressed as means ± standard error of the mean (SEM). $^{***}P<0.001$ vs. Olive–Saline group, $^{\#}P<0.05$ vs. Olive–Morphine group.

Fig. 4



Effect of geranylgeranylacetone [GGA: 500 mg/kg, intraperitoneally (i.p.)] on the development of morphine analgesic tolerance represented by maximal possible effect (MPE) (a) and area under the curve (AUC) (b). GGA given 24 h before each morphine injection accelerated the development of analgesic tolerance and decreased the analgesic efficacy of morphine (n=5). Data are expressed as means ± standard error of the mean (SEM). $^{***}P<0.001$ vs. Olive–Saline group, $^{\#}P<0.05$ vs. Olive–Morphine group.

Fig. 5



Effect of pifithrin-μ [PES: 20 mg/kg, intraperitoneally (i.p.)] on the development of morphine analgesic tolerance represented by maximal possible effect (MPE) (a) and area under the curve (AUC) (b). PES given 30 min before each morphine injection suppressed the development of analgesic tolerance and enhanced the analgesic efficacy of morphine (n=5). Data are expressed as means ± standard error of the mean (SEM). $^{***}P<0.001$ vs. Olive–Saline group, $^{\#}P<0.05$ vs. Olive–Morphine group.

(Fig. 5b). Thus, pifithrin-μ suppressed the development of morphine tolerance, suggesting a role of Hsp70 in the development of morphine analgesic tolerance.

Discussion

Based on our previous studies which showed relationship between Hsp70 in NAc and neuroadaptation associated with morphine dependence, the present study, for the first time, demonstrated a possible role of Hsp70 in PAG in the development of morphine analgesic tolerance in rats. Firstly, chronic morphine injection led to the development of analgesic tolerance and increase of Hsp70 expression in PAG. Secondly, analgesic tolerance to morphine was suppressed by pretreatment with Hsp70 transcriptional inhibitor (KNK437), while promoted by pretreatment with Hsp70 transcriptional inducer (geranylgeranylacetone). Finally, the functional inhibition of Hsp70 substrate-binding domain (SBD) activity by pifithrin-μ slowed down the development of analgesic tolerance to morphine.

Hsp70, also named Hsp72 or HspA1A, is a major member of Hsp70s family mainly functioning as molecular chaperone in the cytosol and nucleus (Evans *et al.*, 2010). The roles of Hsp70 include assisting its protein substrates in folding or refolding, the subcellular transport, the formation and dissociation of complexes and the stabilization against denaturation (Hartl *et al.*, 2011). Gene expression of Hsp70 was reported to be increased markedly by repeated morphine exposures in many brain areas

(Ammon-Treiber *et al.*, 2004). The level of Hsp70 protein was also found to be elevated in brain homogenates and striatum after chronic morphine administration (Joshi et al., 2014, 2015; Yang *et al.*, 2014). Here, we found that Hsp70 protein expression in the PAG was augmented significantly after continuous morphine injection for 4 days and stayed at elevated levels in following injection days. As the PAG has abundant opioid receptors and is important for the development of analgesic tolerance to opioids (Loyd and Murphy, 2009; Fyfe *et al.*, 2010), our results suggest that Hsp70 may participate in the neural adaptive changes of opioid analgesic tolerance.

To further understand the role of Hsp70, we investigated the effect of Hsp70 modulators on the development of opioid analgesic tolerance. Three specific Hsp70 modulators, KNK437, geranylgeranylacetone, pifithrin-μ, were employed in this study, all of which were demonstrated to regulate Hsp70 expression or function in brain or tumor issue after given systemically (Koishi *et al.*, 2001; Fujiki *et al.*, 2003; Chiu *et al.*, 2017). KNK437 and geranylgeranylacetone are Hsp70 transcriptional modulators but in opposite directions. Heat shock factor-I (HSF-I) is an essential element for Hsp70 gene transcription, which is inactivated by binding to constitutively expressed Hsp70 under normal conditions (Kiang and Tsokos, 1998). The association of Hsp70 and HSF-I could be disrupted by metabolic or exogenous insults, and then HSF-I is phosphorylated to initiate Hsp70 gene transcription (Otaka *et al.*, 2007). KNK437 inhibits the phosphorylation of HSF-I and suppresses Hsp70 expression (Koishi *et al.*, 2001), while geranylgeranylacetone facilitates the disassociation of HSF-I and constitutively expressed Hsp70, and promotes the Hsp70 gene transcription (Otaka *et al.*, 2007). Pifithrin-μ is a selective Hsp70 functional antagonist, which interacts with the SBD of Hsp70 and suppresses the binding of Hsp70 to its substrates (Leu *et al.*, 2009).

Firstly, we examined effect of KNK437 on the morphine analgesic tolerance. Inhibition effect of KNK437 on Hsp70 expression *in vivo* was previously observed in brain and tumor tissue (Koishi *et al.*, 2001; Yu *et al.*, 2015). In this study, the decrease of analgesic effect of morphine after repeated morphine exposures was delayed by KNK437 administration. In other words, decrease of Hsp70 expression retarded the development of morphine analgesic tolerance. Then, we further observed effect of geranylgeranylacetone on morphine analgesic tolerance. Geranylgeranylacetone is a widely used antiulcer agent, and its induction effect on Hsp70 expression in brain has been extensively demonstrated (Sinn *et al.*, 2007; Zhao *et al.*, 2013). Our results showed an acceleration of development of analgesic tolerance was caused by geranylgeranylacetone. Together with the effect of KNK437, these data of bidirectional regulation demonstrated that Hsp70 transcription is critically essential in the development of morphine analgesic tolerance.

Besides the transcriptional modulation of Hsp70, we also investigated the effect of functional antagonism of Hsp70 on the development of morphine analgesic tolerance. Pifithrin-μ used in this study is a selective Hsp70 functional inhibitor which does not bind to homologs of Hsp70, such as heat shock cognate protein 70 (Hsc70), binding immunoglobulin protein (BiP) (Leu *et al.*, 2009; Leu *et al.*, 2011; Huang *et al.*, 2013). Our results showed that pifithrin-μ suppressed the development of morphine analgesic tolerance but had no effect on pain threshold of rats. These data suggested that functional participation of Hsp70 is needed for the development of analgesic tolerance to morphine.

In the present study, by transcriptional and functional modulation, we demonstrated that Hsp70 may play an important role in the development of morphine analgesic tolerance. In agreement with our research, Dobashi *et al.* (2010) reported that morphine tolerance is attenuated in mice expressing a mutant BiP, a homolog of Hsp70 which fulfills specific chaperone functions in the endoplasmic reticulum. As only targeting a single mechanism is the major disadvantage of present drugs for opioid analgesic tolerance (Xu *et al.*, 2014), chaperone Hsp70 could be a potential effective therapeutic target because it may assist various tolerance-related proteins in folding and translocating.

Acknowledgments

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Conflicts of interest

There are no conflicts of interest.

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